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Effect of ivermectin on *Trypanosoma brucei brucei* in experimentally infected mice

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ABSTRACT

Background & objectives: Human and livestock African trypanosomiasis, otherwise known as sleeping sickness, is a neglected tropical disease of public health importance in west and central Africa. In view of the adverse side effects of the antitrypanosomal drugs, the relatively few side effects observed in ivermectin use, and because both onchocerciasis and typanosomiasis occur in overlapping foci in Africa, it would be desirable if the ivermectin that has been used successfully on onchocerciasis management could also be used in the control and treatment of trypanosomiasis.

Method: In this study, prophylactic and therapeutic effects of ivermectin (Mectizan) were investigated in albino mice infected with a Nigerian strain of *Trypanosoma brucei brucei.*

Results: A 300 µg/ml/kg dose had the most effective impact because it showed the highest mean survival time of 12 days in both the treatment and prophylactic groups of mice. This dose also enhanced the defence capacity of the treated groups. It also had positive influence on the packed cell volume (PCV) and the state of anaemia in the trypanosome infected mice, hence, improving their survivability.

Interpretation & conclusions: Our report indicates that using the 300 µg/ml/kg dose of ivermectin increases the mean survival period from 5 to 12 days. This suggests that ivermectin could be possibly used in the treatment of trypanosomiasis. Further studies will be required to show whether proper treatment may entail a single dose, as used in this study; an increased number of doses, or combinations with other drugs.

Key words Albino mice; ivermectin; mectizan; treatment; trypanosomiasis

INTRODUCTION

Human and livestock African trypanosomiasis, otherwise known as sleeping sickness, is a neglected tropical disease of public health importance in west and central Africa. It is endemic in 36 African countries[1]. About 50 million people and 48 million cattle are at risk of contracting the disease with estimated annual loss in cattle production of US$ 1–1.2 billion[2]. Human African trypanosomiasis (HAT) cases are caused by the parasites *Trypanosoma brucei gambiense* and *Trypanosoma brucei rhodesiense*, which are indigenous to west and central Africa. Cattle trypanosomiasis also known as Nagana is due to *T.b. brucei*. Both are transmitted by the tsetse fly, *Glossina* sp[3,4].

Nagana causes impaired bovine fertility, reduction in herd size, low milk yields, retarded growth, reduced work output, and a high mortality rate among infected animals[2,5]. Therefore, it has a negative impact on the household incomes of farmers in endemic areas. Animal sleeping sickness is a major threat to animal husbandry and agricultural socioeconomic development in Nigeria[6]. During progress from the first stage of the infection process, the subclinical haemolymphatic phase to the second stage, the meningo-encephalitic phase, the parasites invade the central nervous system (CNS). If not treated adequately, this stage is often 100% fatal[7].

The currently available drugs for trypanosomiasis treatment include pentamidine[8], melarsoprol[9], and eflornithine[10]. These drugs are old, complicated to administer, and are reported to cause severe adverse reactions[11–13]. Suramin was discovered in 1921 and used in the first stage treatment and prophylaxis of African trypanosomiasis. It is also used to treat river blindness (onchocerciasis)[14,15]. In 1941, pentamidine was approved and used for chemoprophylaxis and for the treatment of the first stage of *T.b. gambiense* sleeping sickness. It has also been used to treat leishmaniasis, and the fungus—*Pneumocystis jiroveci*[16]. Additionally, the parasites are evolving resistance to melarsoprol, a drug that is an arsenical derivative with dangerous side-effects[12, 17]. A combination of nifurtimox and eflornithine has shown promising results in the treatment of second stage of African trypanosomiasis[18], but its use is marred by dangerous side-effects. Eflornithine

or diethylfluoromethylornithine (DFMO) is difficult to administer as it requires one slow infusion every six hours for 14 days (56 infusions in total) and its side-effects include seizures, gastrointestinal disorders, and myelosuppression[19]. The drug nifurtimox, an orally administered drug originally used in the treatment of Chagas' disease (caused by *T. cruzi*), is reported to cause neurological and gastrointestinal disorders that increase with the duration of intake[10]. With the magnitude of the threats to humans; game and domestic animals; and the socio-economic burden of African trypanosomiasis, combined with the problems posed by the existing drugs[20], there is an urgent need to find safer and more effective antiparasitic drugs.

Ivermectin (Mectizan) is a semi-synthetic macrolide that has broad-spectrum drug activity. It is an effective and well-tolerated microfilaricidal drug. It has also emerged as the drug of choice for large-scale treatment of onchocerciasis caused by *Onchocerca volvulus*. While a single dose of ivermectin markedly reduces skin microfilarial loads up to 12 months, with a transient fall in microfilarial level[21], repeated doses of ivermectin are reported to lower the incidence of microfilaremia. Ivermectin is widely available through the Onchocerciasis Control Programme (OCP) in Nigeria and other west African countries[22] and it is currently the most accepted method for control of recrudescence of infection in an area where the parasite reservoir has been virtually eliminated by vector control[23]. Ivermectin is also an effective suppressor of inflammation[24]. The reported side-effect of ivermectin include; fever, itching skin, joint/muscle pain, painful and tender glands in neck, armpits, or groin, rapid heartbeat, eye or eyelid irritation, pain, redness, or swelling, nausea, pallor, and transient pain and numbness in the affected extremity[25], with recovery within 12 h[26].

T. brucei brucei, the cause of Nagana, was chosen as the model for this study because it mimics HAT, which is caused by *T.b. rhodesiense* and *T.b. gambiense*. This murine model has been widely used in the evaluation of the efficacy of trypanosomiasis chemotherapeutic agents[27].

Ivermectin is chosen for this research because it is readily available through the Onchocerciasis Control Programs (OCP) in different areas of Africa endemic to trypanosomiasis and onchocerciasis. It has been demonstrated previously that both *T.b. brucei* and *O. volvulus* could be susceptible to the same drug, such as suramin[14,15]. In view of the adverse side-effects of the antitrypanosomal drugs, the relatively reversible side-effects of ivermectin use, and the existence of both the onchocerciasis and typanosomiasis in overlapping foci in Africa, it would be desirable if a simple, effective, user-friendly drug such as ivermectin[26] could be used in the treatment of trypanosomiasis.

The objective of this study was to determine the effect of therapeutic ivermectin on African trypanosomiasis. A group of mice was treated prophylactically with ivermectin before exposure to *T.b. brucei,* to simulate the OCP in Nigeria. Another group was exposed to *T.b. brucei* before being treated with ivermectin to determine the therapeutic effect of ivermectin on trypanosomiasis. The control group comprised of mice uninfected with *T.b. brucei* but treated with distilled water (positive control) and mice infected with *T.b. brucei* and left untreated (negative control).

METHODS

Test animals

Adult male Swiss albino mice, 6–8 wk of age, and weighing between 24 and 30 g, were used for this study and were obtained from the Laboratory Animal Centre of College of Medicine, University of Lagos, Nigeria. These were allowed to acclimatize for a week in the Animal Unit of the Department of Microbiology before the study. They were kept in cages lined with wood shavings and cleaned twice a week. The cages were also provided with water bottles to supply drinking water *ad libitum* to the mice. The mice were fed regularly on standard commercial mouse cubes (Pfizer Nigeria PLC, Ikeja, Nigeria). Each cube contained 21% protein, 3.5% (minimum) fat, 6% (minimum) fibre, 0.8% calcium, 0.8% phosphorous, maize, wheat middling, fish meal, groundnut cake, dried grains, brewer's yeast, bone meals, oyster shell, salt, antioxidant and antibiotics.

Test parasites

The *T.b. brucei* used for this study was obtained from Dr V.I. Okochi, Department of Biochemistry, University of Lagos, College of Medicine, Idi-Araba Lagos, Nigeria. The parasite was maintained in the laboratory by passage of blood from infected mice to uninfected ones. The tip of the tail of an infected mouse was cut with a sterile surgical blade; the tail was milked upwards to obtain drops of blood, which were diluted in sterile normal saline solution. Each of the recipient animals was inoculated intraperitoneally with 3.6×10^3 *T.b. brucei* cells.

Drug concentration determination

One tablet contains 6 mg ivermectin and each dose was determined based on the weight (kg) of the mouse. The treatment range of 200 to 400 µg/ml/kg was selected because the human body can tolerate up to 600 µg/ml/kg although the standard dose used in treating *O. volvulus* infection in human is 150 µg/kg annually[15,26].

(1) The different doses of ivermectin were prepared using the following formula:

Volume of water for dissolution = Weight (µg)/ required concentration (µg/ml)[28]

(2) To calculate the volume drug administered to each animal, the following formula was used[29];

Volume (ml) = Weight (kg) × Dose (mg/kg)/ Concentration (mg/ml)

Parasite inoculation/drug administration

All parasite inoculations were done via the intraperitoneal route and for the drug administration, each mouse was orally given the dissolved drug suspension using a blunt medicut needle and syringe. The preparation was administered as a single dose and the animals were treated without food.

Design of the study groups

In all, 32 animals were randomly allocated into the following groups; 2 control groups, 3 prophylactic groups, and 3 treatment groups, with 4 mice per group. Each group of mice was housed separately. Prior to the exposure of the mice to trypanosomes, groups were either exposed to various concentrations of ivermectin (200, 300 and 400 µg/ml/kg body weight), or a volume of water equal to the volume of the drugs. These controls were used to determine if water or the drugs were toxic and caused the death of the animals in the absence of the infection due to the route of administration.

Control group

Group A: Uninfected and untreated (control); 4 mice; and Group B: Infected and untreated; 4 mice.

Prophylactic group

Group D: Infected and treated with ivermectin 200 µg/ml/kg body weight, 4 mice; Group E: Infected and treated with ivermectin 300 µg/ml/kg body weight, 4 mice; and Group F: Infected and treated with ivermectin 400 µg/ml/kg body weight, 4 mice.

The 12 mice were treated with respective dosages of ivermectin and after 3 days of post drug administration, they were inoculated intraperitoneally with approximately 1.0×10^5 *T.b. brucei* parasites. The peripheral parasitemia in their tail blood were determined daily after the treatment and a mouse was assumed to be protected if parasites were not detected from the tail blood.

Treatment group

Group G: Infected and treated with ivermectin 200 µg/ml/kg body weight, 4 mice; Group H: Infected and treated with ivermectin 300 µg/ml/kg body weight, 4 mice; and Group I: Infected and treated with ivermectin 400 µg/ml/kg body weight, 4 mice.

The 12 mice total were inoculated intraperitoneally with approximately 1.0×10^5 *T.b. brucei* parasites and were subsequently treated with 200 µg/ml/kg, 300 µg/ml/kg, or 400 µg/ml/kg of ivermectin after parasitemia was observed in their tail blood. The peripheral parasitemia in their tail blood were determined daily after treatment and the mouse was assumed cured if parasites were not detected from the tail blood.

Determination of degree of parasitemia

The patency of *T.b. brucei* was determined by wet film examination of blood from a tail snip. After confirming the presence of the parasite, thin smears were prepared to estimate the number of parasites per 1000 red blood cells (RBCs).

Blood film preparation

A drop of blood was taken from the tail vein of the infected mice and placed at a distance of about an inch from one end of the grease free microscopic slide. A spreader was lowered at an angle of 45° in front of the blood drop. The spreader was then drawn backwards until the blood was touched making it to spread along the line of contact between the spreader and the horizontal slide. The spreader was pushed at a uniform speed drawing the blood into a thin film. The slide was air-dried.

Staining of blood film

The dried thin blood film was fixed in methyl alcohol for 60 sec and stained for 40 min with diluted stock Giemsa stain (1 part Giemsa to 10 parts buffered water). The slides were rinsed with buffered water until the stain colour did not run out noticeably from the film. The slide film was placed in an angle to dry and was examined under an oil immersion (×100) objective lens and the number of parasites per 1000 red blood cells were counted. The degree of parasitemia was expressed as a percentage of the total number of RBCs counted.

$$\text{Percentage parasitemia} = \frac{\text{No. of parasites counted}}{\text{1000 RBCs}} \times 100$$

Packed cell volume (PCV) analysis

Packed cell volume (PCV) was determined using the

standard micro-haematocrit method. Blood was collected from the tail snip by capillary action in 100 µl micro-haematocrit tubes coated with heparin-sodium and centrifuged at 10,000 rpm for 5 min using a Hawksley micro-haematocrit centrifuge (Hawksley, UK). PCV was read with a Hawksley micro-haematocrit reader and determined on Day 1, Day 3 and Day 6 post-infection because the mean survival period of the untreated group of mice was 5 ± 1 days. The ethical clearance for the use of animals for this research was granted by the Research Ethics Committee of the College of Medicine, University of Lagos, Nigeria.

Statistical analysis

The significance of difference between means and the significance level at $p < 0.05$ was determined using one-way analysis of variance (ANOVA) calculator (Online software)[30].

RESULTS

Control group

No death was observed in the Group A mice (positive control), which were neither treated nor infected. However, all the mice in Group B, infected and without treatment, died within 7 days after infection with a mean survival time of 5 ± 1 days as shown in Figs. 1 & 2. The pre-patent period (the interval between infection and detection of parasites in the animal's blood) for *T.b. brucei* in mice was found to be between 2 and 3 days. The stage 1 signs of the disease such as reduced food and water intake were observed. Typical signs of stage 2 of African trypanosomiasis such as reduced activity, anaemia (low PCV) (see Figs. 3 & 4), loss of weight, sleepiness and death were observed in Group B mice (negative control). There was no significant difference at $p > 0.05$, in the PCV of all the groups including the control groups on Day 1.

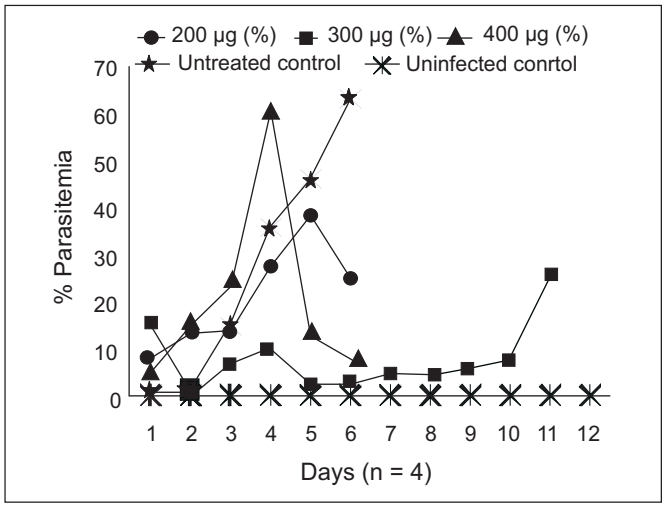

Fig. 1: Mean parasite count in the Treatment group of mice.

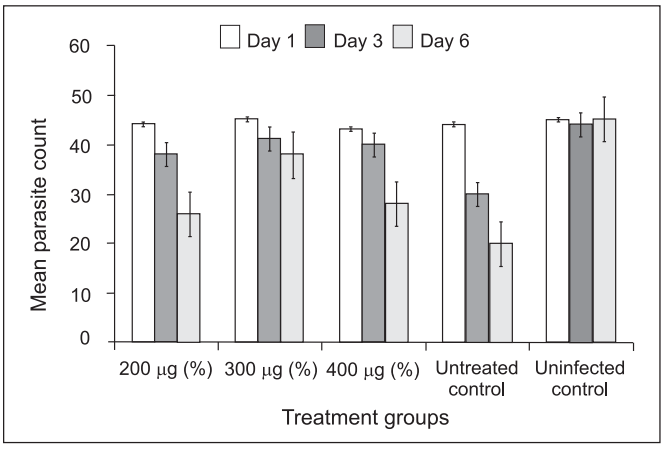

Fig. 2: Mean parasite count in the Prophylactic group of mice

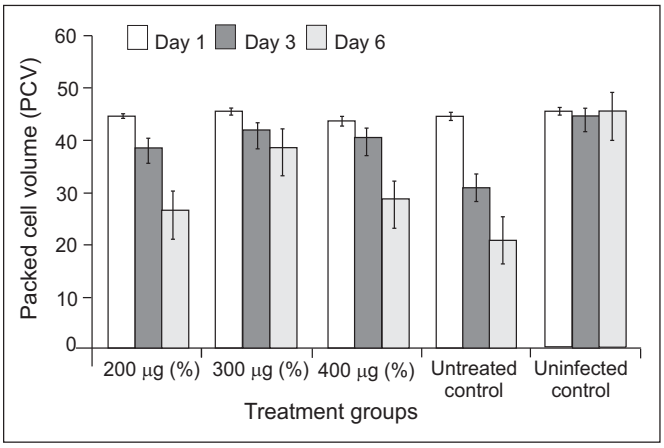

Fig. 3: Mean packed cell volume (PCV) in the Treatment group of mice.

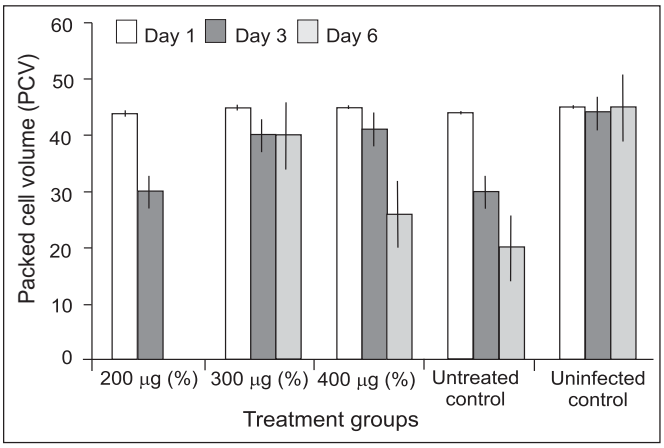

Fig. 4: Mean packed cell volume (PCV) in the Prophylactic group of mice.

Prophylactic group

This comprised the group of mice treated with

ivermectin 200, 300 and 400 µg/ml/kg, three days prior to infection with *T.b. brucei.* All the mice treated with 200 and 400 µg/ml/kg developed parasitaemia on Day 2 post-inoculation with average parasitemia of 0.3 and 0.2%, respectively. The entire 300 µg/ml/kg group developed parasitemia on Day 3 post-inoculation. By Day 5 post inoculation, all the mice in the 200 µg/ml/kg group had died (Fig. 2). The longest surviving mouse was seen in the 300 µg/ml/kg group. It survived up to 11 days with a parasitemia of 52.1%. The mean survival period of 300 µg/ml/kg group was significantly different (p <0.05) from the other treatment concentrations. In the 400 µg/ml/kg treated group, the mice started dying after Day 4 and the last set died on Day 7 with an average parasitemia of 10.3% on Day 6. In Fig. 3, it was observed that the mice in group 300 µg/ml/kg had a higher level of parasitemia but they survived longer than those in 400 µg/ml/kg group which had a lower level of parasitemia. The therapeutic effect of the 300 µg/ml/kg was significantly different from the rest of the concentrations used at p <0.05.

Packed cell volume (PCV)

A proportional reduction in PCV signifying anaemia was observed as the level of parasitemia was increasing. There was no change in the uninfected mice group, while there was a sharp decrease in the untreated infected control mice (negative control). The starting PCV was 44% on Day 1 prior to infection and the PCV dropped to 20% by Day 6 post-inoculation after which the mice died. As shown in Fig. 4, there was no PCV reading for the 200 µg/ml/kg group because all the mice had died on Day 6 before the reading was taken. The visualization of the effect of trypanosome infection on PCV in mice is further illustrated in Fig. 5. The figure shows a progressive reduction in PCV in the untreated control group and the 200 µg treatment group. However, the mice treated with 300 µg concentration sustained their PCV, with final values on Day 6 closest to the initial values.

Treatment group

The mice in this group were infected with *T.b. brucei* and treated with ivermectin 200, 300 and 400 µg/ml/kg three days after the infection has been confirmed by observation of trypanosomes in the tail blood of the infected mice. As illustrated in Fig. 1, mice treated with 200 and 400 µg/ml/kg survived up to Day 6 post-treatment and by Day 7 all the mice had died. But for the 300 µg/ml/kg group, there was a reduction in parasitemia from 10% on Day 4 to 2.8% on Day 5 and 2.2 % on Day 6. Mice in this group were also still agile, but the level of parasitemia

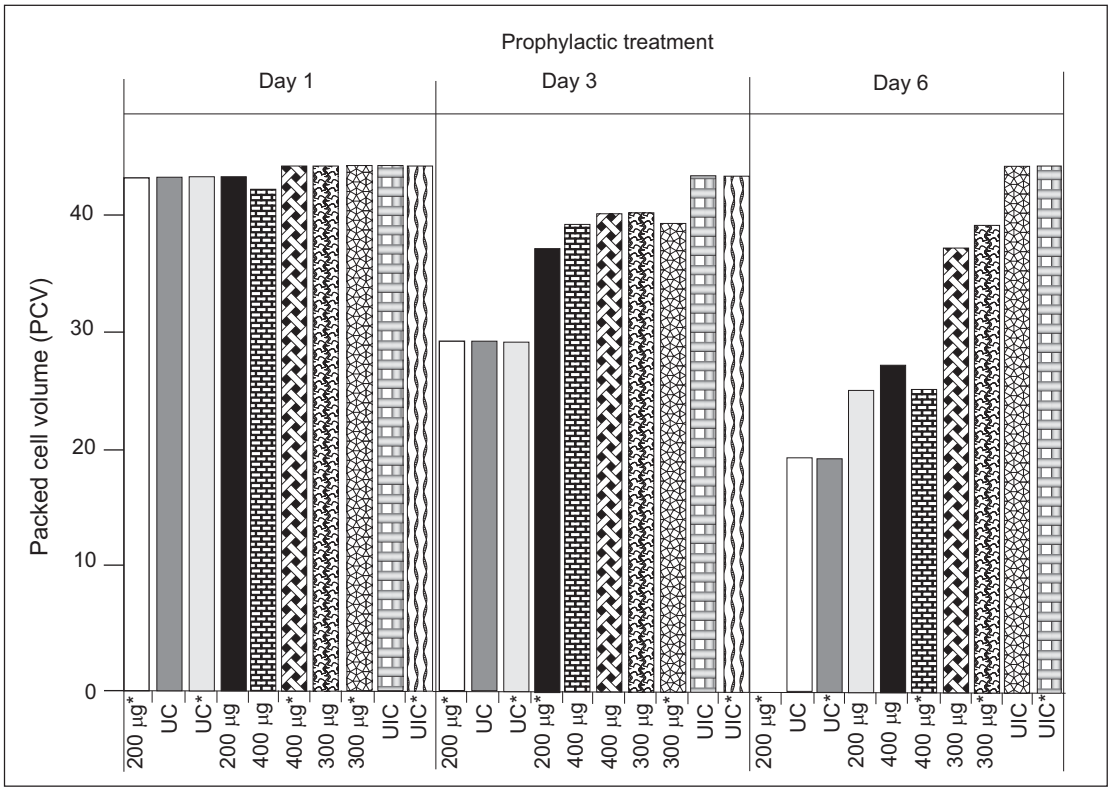

Fig. 5: Combined data of the mean packed cell volume (PCV) of both the Treatment and Prophylactic groups of mice infected with *T. b. brucei* and treated with ivermectin; *Prophylactic group data; UC = Untreated control; UIC = Uninfected Control

started abruptly rising again from Day 7, with 4% parasitemia, culminating with a parasitemia of 25% on Day 11, after which the last two mice died on Day 12.

Table 1 illustrates that there are significant differences in the response to different treatment concentrations based on the values (mean ± SEM) of parasite count per 1000 RBCs (%), with the greatest in mice infected with *T.b. brucei* and treated with ivermectin 3 days post-inoculation. As also observed in the prophylactic group, the PCV volume was reduced as the level of parasitemia was increasing. This is shown in Fig. 3, where the PCV of the 200 µg/ml/kg group had dropped from 44% on Day 1 prior to infection to 26% on Day 6 post- infection. No significant drop in PCV was noticed in the 300 µg/ml/kg group within the 6-day test period. The 400 µg/ml/kg group had dropped from 44 to 28% over the same period. There was a significant difference in PCV values between the 300 µg/ml/kg group and the untreated group ($p < 0.05$). However, there was a significant PCV difference between the untreated group and the 300 µg/ml/kg group on Day 1, 3 and 6 post-infection ($p < 0.05$).

DISCUSSION

The results show that the pre-patent period of *T.b. brucei* is between 2 and 3 days, which is in concert with Turay *et al*[31]. Ivermectin appears to have no prophylactic effect against *T.b. brucei* since the patent period in the prophylactic group was not delayed more than those in the negative control group ($p < 0.05$). However, this could be because the blood level of the drug may have reduced over time. Perhaps if the doses were given at more regular intervals as in malaria prophylaxis, the prophylactic activity may have been enhanced[32].

The effect of the 300 µg/ml/kg dose was significantly different from the other doses at $p < 0.05$. It appears to be the most effective because it has the highest mean survival time of 12 days in both the treatment and prophylactic groups of mice. It enhanced the defence capacity of the treated mice group, especially at the early stages of the infection. This dose also had a positive influence on the PCV and the state of anaemia in the trypanosome-infected mice and hence their ability to survive for a longer time. This observation can be clearly visualized in Fig. 5. Other studies have demonstrated that any substance which has the ability to improve the haematological parameters of any organism infected with trypanosomes will increase its resilience to developing trypanosomiasis[33]. Conversely, reduction in PCV and anaemia will result in complications with African trypanosomiasis[31,34] which increases the morbidity and mortality rates of the disease. The tissue invasion by *T.b. brucei* contributes adversely to the reduction in PCV seen in trypanosomiasis. The parasitemia crises compounded by the early onset of anaemia observed in this study have been observed by other researchers: Karori *et al*[27] and Jennings *et al*[34] working with *T.b. brucei* in mice. The low PCV observed in this study may have been caused by the coating of the erythrocytes from infected mice with immunoglobulin M, forming immunoglobulin M-antigen complexes and endotoxin induction. This would eventually result in the lysis of red blood cells and suppression of the immune system[35].

Table 1. Mean ± SEM of parasite count per 1000 RBCs (%) in the Treatment group of mice

Treatment	Day 1	Day 3	Day 6
200 µg (%)	7.5 ± 0.081	13.6 ± 0.14	25 ± 1.825
300 µg (%)	15 ± 0.081	6.3 ± 0.216	2.2 ± 0.182
400 µg (%)	5.2 ± 0.081	23.3 ± 0.244	8 ± 0.816
Infected and treated control	No parasite	14.8 ± 0.216	63 ± 2.160
Uninfected and untreated control	DNR $p < 0.001$	DNR $p < 0.001$	DNR $p < 0.001$

DNR—Did not run since the mice were not infected.

A relapse was observed in the 300 µg/ml/kg dose on Day 4 after a brief period of recovery. It could be inferred from this that on Day 4, the infection had entered into the stage 2 phase with CNS involvement[7]. This could be the result of diminished concentration of ivermectin in the CNS, hence, the resurgence of parasitemia, and death shortly afterwards. Encephalopathy associated microhaemorrhages[36] have been suggested as the cause of death due to relapse in trypanosomiasis. Similar relapses have been reported in other animals infected with *T. brucei* species[37,38]. Additionally, the sudden upsurge in parasitemia in the treated group, despite the ability of ivermectin to suppress inflammation[24], may suggest a total breakdown of the host's defence systems.

CONCLUSIONS

The present report indicates that ivermectin increased the mean survival period from 5 days to 12 days using the 300 µg/ml/kg dose. This suggests that ivermectin could be possibly used in the management of trypanosomiasis. Further studies are required to show whether proper treatment may entail a single dose, as used in this study; an increased number of doses, or combinations with other drugs.

Competing interests: The authors report no conflicts of interest.

Authors' contributions

The authors conceived and designed the study and have read and approved this manuscript. This paper is unique and not under consideration by any other publication and has not been published elsewhere.

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